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Bradley J. Berger and Marvin H. Knodel
Defence R&D Canada – Suffield

Technical Report
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Abstract

The aminotransferases involved in the final step of methionine recycling from methylthioadenosine have been examined in the gram-positive bacterium *Bacillus subtilis*. Homogenates of this bacterium were able to convert ketomethiobutyrate to methionine, utilising leucine, isoleucine, valine, phenylalanine, tyrosine, and alanine as preferred amino donors. Unlike other organisms examined to date in this context, *B. subtilis* was found to contain no aspartate aminotransferase or tyrosine aminotransferase sequences with structural homology to subfamily Ia aminotransferases. Instead, in *B. subtilis*, the six putative homologues of aspartate aminotransferase were found to be members of the If subfamily. Five of these six sequences were cloned and expressed, with only the ykrV gene product capable of producing methionine from ketomethiobutyrate. However, this enzyme only catalysed the reaction using glutamine as an amino donor. Two putative branched-chain amino acid aminotransferases from family III were also cloned and expressed, and both were found to produce methionine from ketomethiobutyrate using branched-chain and aromatic amino acids. Of these two enzymes, the ybgE gene product was the most active and had kinetic constants consistent with it being the enzyme responsible for the majority of methionine regeneration in this organism. The ybgE gene product could be inhibited uncompetitively by the aminooxy compound canaline, with a Ki of 48 μM. In addition, canaline inhibited the in vitro growth of *B. subtilis* in minimal medium with an IC_{50} of 37 μM and an MIC of 500 μM. Growth inhibition in nutrient broth was negligible.

Résumé

Les transaminases présentes dans la phase finale du recyclage de la méthionine à partir de la méthylthioadénosine ont été examinées dans la bactérie gram positive *Bacillus subtilis*. Les homogénats de cette bactérie ont été capables de transformer le kétométhiobutyrate en méthionine, en utilisant, de préférence, la leucine, l'isoleucine, la valine, la phénylalanine, la tyrosine, et l'alanine comme donneurs amines. À la différence des autres organismes qui ont été examinés dans ce contexte jusqu'à présent, on a trouvé que le *B. subtilis* ne contenait pas de séquences de transaminases d'aspartate ou de transaminases de tyrosine ayant une homologie structurelle avec la sous-famille de transaminases Ia. À la place, on a trouvé que dans le *B. subtilis*, les six homologues putatifs de transaminases d'aspartate étaient membres de la sous-famille If. Cinq de ces six séquences ont été clonées et exprimées et seul le produit génétique ykrV a été capable de produire de la méthionine à partir de kétométhiobutyrate. Cet enzyme n'a cependant catalysé que la réaction utilisant la glutamine comme donneur amine. Les deux transaminases d'aminoacides de chaîne ramifiée appartenant à la famille III ont aussi été clonées et exprimées et on a trouvé qu'elles étaient toutes les deux capables de produire de la méthionine à partir de kétométhiobutyrate, en utilisant les aminoacides aromatiques et de chaîne ramifiée. Le produit génétique ybgE était le plus actif de ces deux enzymes et possédait des constantes cinétiques qui correspondaient au fait qu'il est l'enzyme causant la majorité de la régénération de la méthionine, dans cet organisme. Le produit génétique ybgE a pu être inhibé sans concurrence par le composé aminooxy canaline avec une Ki de 48 μM. De plus, la canaline a inhibé la croissance in vitro de la bactérie *B. subtilis* dans un milieu minimum avec une CI_{50} de 37 μM et une CMI de 500 μM. L'inhibition de la croissance dans le bouillon nutritif était négligeable.

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Executive summary

In order to synthesize the polyamines required to complete cell replication, most organisms have a unique pathway for recycling the amino acid methionine, which is consumed during polyamine biosynthesis. The final step of this pathway is the conversion of ketomethiobutyrate to methionine by an aminotransferase. In previous studies, inhibitors active against the enzymes that catalyse this reaction have been demonstrated as potent antimalarials in vitro. As there is a constant requirement for novel antimicrobials against biological warfare agents, a similar approach has been undertaken in *Bacillus subtilis* as a model for *B. anthracis*. Unlike other organisms examined to date, which utilise aminotransferases of the Ia subfamily to catalyse methionine regeneration, *B. subtilis* was found to contain no gene sequences with homology to the Ia subfamily. The nearest homologues were all found to be members of the If subfamily. Five of these putative aspartate aminotransferase homologues were cloned, expressed, and examined for activity. Only one, the ykrV gene product, was found to catalyse methionine regeneration. However, this enzyme only did so using glutamine as an amino donor for the reaction. *B. subtilis* cellular homogenates, conversely, were found to use leucine, isoleucine, valine, phenylalanine,and tyrosine as amino donors. Therefore, two putative branched-chain amino acid aminotransferases were identified, cloned, expressed, and characterised from *B. subtilis*. Both of these enzymes, which are members of the aminotransferase family III, were found to catalyse methionine regeneration with an amino donor preference reminiscent of the cellular homogenates. Of these two enzymes, the ybgE gene product was the most active, and represents the catalyst of the majority of methionine regeneration in the organism. The ybgE gene product was effectively inhibited by the aminooxy compound canaline. This compound was also found to be a potent inhibitor of *B. subtilis* growth in vitro in minimal medium, but not in nutrient broth. Therefore, the enzyme responsible for methionine regeneration has been identified in *B. subtilis*, and it has been demonstrated as a suitable target for therapeutic intervention.

Berger, B. J., and Knodel, M. H. 2002. Characterisation of potential antimicrobial targets in *Bacillus spp*. I. Aminotransferases and methionine regeneration in *Bacillus subtilis*. TR2002-048. Defence R&D Canada – Suffield.

Sommaire

Afin de synthétiser les polyamines requises pour compléter la duplication de cellules, la plupart des organismes utilisent un chemin unique pour recycler les aminoacides de méthionine qui sont consommés durant la biosynthèse des polyamines. La phase finale de ce chemin consiste en la conversion du kétométhiobutyrate en méthionine, par une transaminase. Les études précédentes ont montré que les inhibiteurs actifs contre les enzymes qui catalysent cette réaction sont des antipaludiques in vitro puissants. Puisque de nouveaux antimicrobiens contre les agents de guerre biologiques sont constamment en demande, on a entrepris une démarche semblable pour le *B. subtilis* comme modèle pour le *B. anthracis*. À la différence des autres organismes qui ont été examinés jusqu'à présent et qui utilisent les transaminases de la sous-famille Ia pour catalyser la régénération de méthionine, on a trouvé que le *B. subtilis* ne contenait pas de séquence de gènes ayant une homologie avec la sous-famille Ia. On a trouvé que les homologues les plus proches appartenaient tous à la sous-famille If. Cinq de ces homologues putatifs de transaminases d'aspartate ont été clonés, exprimés et leur activité à été étudiée. On a trouvé qu'un seul, le produit génétique ykrV, catalysait la régénération de méthionine et que cet enzyme ne parvenait cependant à provoquer la réaction qu'en utilisant la glutamine comme donneur amine. Par contre, on a trouvé que les homogénats cellulaires de *B. subtilis* utilisaient la leucine, l'isoleucine, la valine, la phénylalanine et la tyrosine comme donneurs amines. Par conséquent, deux transaminases aminoacides putatives de chaîne ramifiée ont été identifiées, clonées, exprimées et caractérisées à partir du *B. subtilis*. On a trouvé que ces deux enzymes qui sont membres de la famille transaminase III catalysent la régénération de méthionine avec une préférence pour les donneurs amines qui rappellent les homogénats cellulaires. Le produit génétique ybgE était le plus actif des deux enzymes et le catalyseur de la majorité de la régénération de méthionine, dans l'organisme. Le produit génétique ybgE a été inhibé efficacement par le composé aminooxy canaline. On a aussi trouvé qu'il était un inhibiteur puissant de la croissance in vitro du *B. subtilis* dans un milieu minimum mais que ce n'était pas le cas dans un bouillon nutritif. En conclusion, on a déterminé l'enzyme causant la régénération de méthionine dans le *B. subtilis* et montré qu'il est une cible apte à l'intervention thérapeutique.

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Introduction

Methionine (Met) is a key amino acid required for protein synthesis, biological methylation, and polyamine biosynthesis. This latter function is particularly important in rapidly growing cells, such as cancer cells and most bacteria and parasites, which must continually synthesize polyamines in order to replicate their DNA [1]. The de novo production of Met is energetically expensive and requires aspartate, ATP, NADPH, succinyl coA, cysteine or H_2S, and 5-methyltetrahydrofolate. In addition, many organisms lack the ability to make Met and rely on exogenous sources. For these reasons, Met bioavailability is limiting and tightly controlled. Almost all organisms examined to date, including those that can synthesize Met, have recycling pathways that can regenerate the amino acid from metabolic by-products.

In the production of spermidine from putrescine or spermine from spermidine, Met is consumed (in the form of decarboxylated S-adenosylmethionine) in a one-to-one stoichiometry (see Figure 1). The end-product of this reaction is methylthioadenosine, which can be recycled to adenine and Met via a unique pathway which has been found in organisms ranging from bacteria to mammals (Figure 1) [2-6]. Studies in cancer cells, bacteria, malaria, and trypanosomes have demonstrated that interference with this Met regeneration pathway leads to the death of rapidly growing cell types. In these studies, the target enzyme was methylthioadenosine phosphorylase or methylthioadenosine nucleosidase, which represents the first step in the bioconversion to Met [2,7-9].

This laboratory has been involved for some time in the investigation of the final step of the Met regeneration pathway, where ketomethiobutyrate (KMTB) is converted to Met via an aminotransferase. The exact enzyme catalyzing this final step has been examined in a number of organisms, including *Trypanosoma brucei brucei*, *Crithidia fasciculata*, *Giardia intestinalis*, and *Plasmodium falciparum* [10,11]. In these organisms, aspartate aminotransferase (AspAT) was found to be the enzyme responsible. In vitro growth inhibition studies have also shown that inhibitors of the parasite AspAT have a cytocidal effect on the cells. In the case of *P. falciparum*, inhibitors were uncovered which had an IC50 in the 200-500 nM range [12]. In studies on the gram-negative bacterium *Klebsiella pneumoniae*, tyrosine aminotransferase (TyrAT) was found to catalyse the reaction [13]. Both the parasite AspATs and the bacterial TyrAT were found to be members of the same subfamily of aminotransferases (subfamily Ia; Figure 2). This subfamily consists of eukaryotic AspATs, gram-negative bacterial AspATs, and bacterial TyrATs, and its constituents tend to have fairly broad substrate specificities [14].

During these previous studies it became clear, using aminotransferase sequences available from completed genome projects, that gram-positive bacteria and archaeabacteria have no members of the aminotransferase Ia subfamily (see below). The few AspATs that have been characterised in these organisms, such as one from *Bacillus subtilis* [15] and one from *Sulfolobus solfataricus* [16], are members of the If subfamily. As it is non-pathogenic, easy to manipulate, and has had its entire genome sequenced, *B. subtilis* acts as a convenient metabolic model for the initial stages of biochemical questions relating to *B. anthracis*. Conclusions drawn from studies in *B. subtilis* are then easily confirmed in more focussed

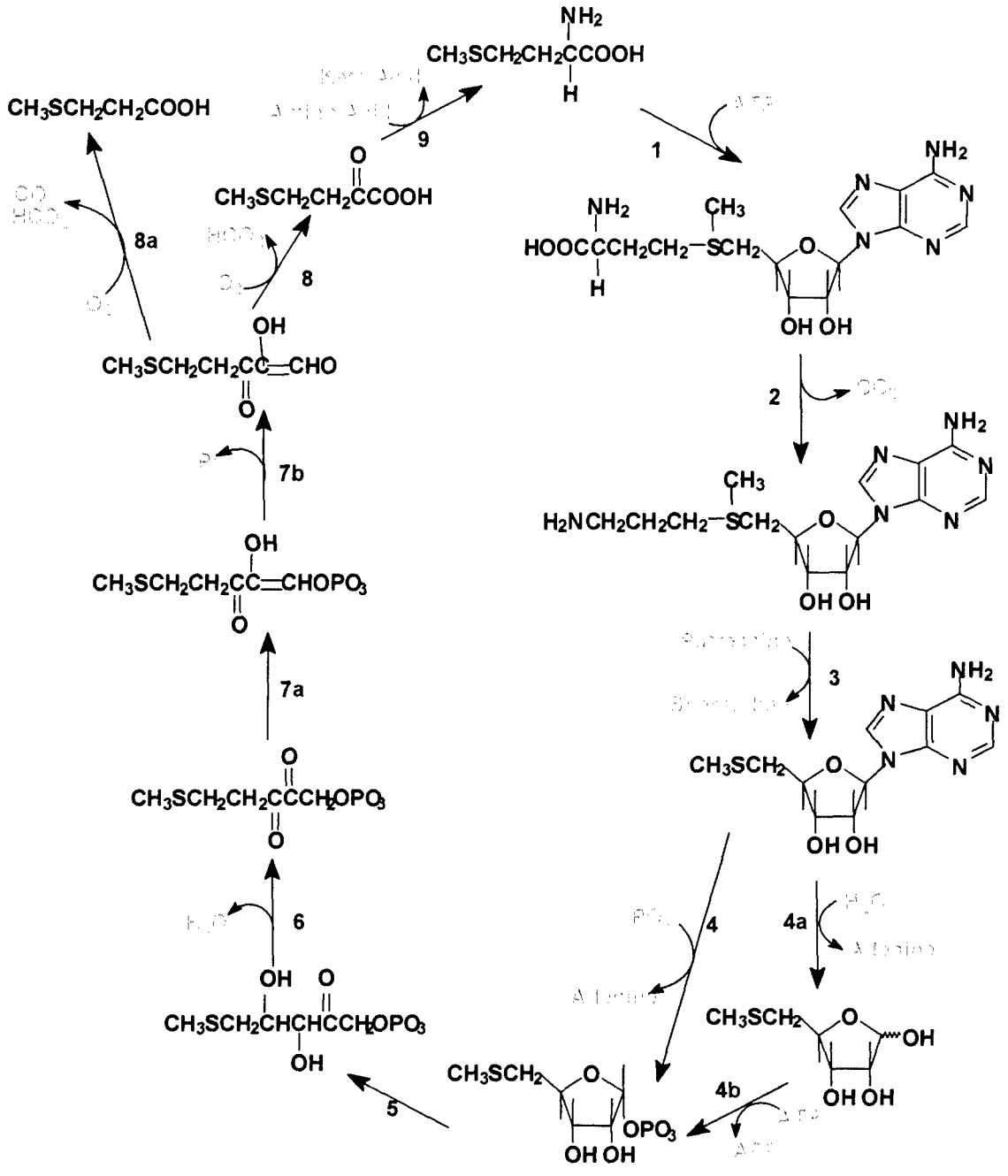

Figure 1. *The Met regeneration pathway. The labelled enzymes are: 1, S-adenosylmethionine synthetase; 2, S-adenosylmethionine decarboxylase; 3, spermidine/spermine synthetase; 4, methylthioadenosine phosphorylase; 4a, methylthioadenosine nucleosidase; 4b, methylthioribose kinase; 5, unidentified isomerase; 6, unidentified dehydratase; 7, E-1 bifunctional enolase-phosphatase; 8, nonenzymatic or via E-3 dioxygenase; 8a, E-2 dioxygenase; 9, various aminotransferases.*

examinations of *B. anthracis*. In this study, we have determined the phylogenic relationship of the AspATs from *B subtilis* 168, and have cloned, functionally expressed, and characterised these enzymes in regards to Met regeneration. In addition, based on the amino acid preference shown by bacterial homogenates in catalyzing the conversion of KMTB to Met, we have also cloned, expressed, and characterised selected members of the family III of aminotransferases. It was discovered that the final step of Met recycling in *B. subtilis* was catalysed by one branched-chain amino acid aminotransferase (BCAT). The effect of a prototypic inhibitor on this enzyme was also investigated.

Materials and Methods

Cells and Reagents

Bacillus subtilis 168 (ATCC 23857) was obtained from the American Type Culture Collection (Manassas, VA, USA), and was routinely cultured in Nutrient Broth at 30°C with agitation at 250 rpm. For selected experiments, the cells were grown in a liquid minimal medium consisting of 0.5% w/v glucose/57 mM K_2SO_4/310 mM K_2PO_4/220 mM KH_2PO_4/17 mM sodium citrate/4 mM $MgSO_4$/1.2 mM tryptophan/68.4 mM glutamine/74 µM $FeCl_3$/5.9 µM $MnSO_4$/187 µM $CaCl_2$/62 µM $ZnCl_2$/13 µM $CuCl_2$/13 µM $CoCl_2$/12 µM Na_2MoO_4 [17].

Subcellular Homogenates

Cells in late-log or stationary phase were harvested by centrifugation at 3500 x g for 20 min and 4°C. The cell pellets were resuspended in a minimal volume of 25 mM PO_4 pH 7.8/120 mM KCl/2.5 mM KG/1.0 mM DTT/0.2 mM pyridoxal phosphate/protease inhibitors (Complete Tablets; Roche Biomedical; Laval, QB, Canada), lysozyme added to 0.3% final w/v, and then incubated on ice for 60 min. The samples were then sonicated on ice and dialysed against 10 mM HEPES pH 7.4/1.0 mM EDTA/1.0 mM DTT at 4°C. After dialysis, the samples were briefly resonicated on ice, and stored at 4°C for enzyme assays. For long term storage, glycerol was added to 20% final v/v and the samples kept at –20°C.

The Met regeneration activity in the *B. subtilis* homogenate was semipurified by ion-exchange chromatography over a 2.6 x 12.5 cm DEAE-Sepharose FF column (Amersham Pharmacia; Baie d'Urfe, QB, Canada). The column was equilibrated with 10 mM HEPES pH 7.4/1.0 mM EDTA/1.0 mM DTT and was eluted with a linear gradient of 0 – 3 M KCl in equilibration buffer. The active fractions were pooled and dialysed against 10 mM $NaPO_4$ buffer pH 7.4/1.0 mM EDTA/1.0 mM DTT at 4°C, and then loaded onto a 1.6 x 9.5 cm hydroxylapaptite column (E. Merck; Darmstadt, Germany). The column was equilibrated with 10 mM PO_4 pH 7.1/1.0 mM DTT and was eluted with a linear gradient to 500 mM PO_4 pH 7.1/1.0 mM DTT. The active fractions were pooled and dialysed against 10 mM HEPES pH 7.4/1.0 mM EDTA/1.0 mM DTT, and then loaded onto a 0.5 x 5 cm MonoQ column (Amersham Pharmacia). The column was equilibrated and eluted as described for the DEAE column. The active fractions were pooled and concentrated using a 30 KDal molecular weight cut-off filter. Throughout purification, the columns were kept at 4°C, and column fractions were

assayed for tyrosine:KMTB aminotransfer using a modified Diamondstone assay as previously described [10].

Enzyme Assays

Aminotransferase activities were assayed by an HPLC method [11]. Ten µl of subcellular homogenate or a variable volume of recombinant enzyme was added to 100 µl of substrate mix (100 mM PO_4/50 µM PLP/various concentrations of amino acid/various concentration of keto acid) and incubated for 30 min at 37°C. The samples were then stored at –20°C until analysis by HPLC as described below. AspAT activity was assayed using 2.0 mM aspartate /1.0 mM KG in the substrate mix, while BCAT activity was assayed using 2.0 mM valine, isoleucine, or leucine/1.0 mM KG. Met regeneration was screened using 2.0 mM ADEFGHIKLNQRSTWY/1.0 mM KMTB in the substrate mix. The range of effective amino donors for Met formation was determined by using 2.0 mM individual amino acid/1.0 mM KMTB in the substrate mix. For the determination of Michaelis-Menton constants, the substrate mixes contained 0.1-10 mM of substrate and 5 mM or 10 mM of the cosubstrate. The inhibition constant for canaline was determined by adding 0, 1.0, 5.0, 10, 50, or 100 µM canaline to enzyme incubations containing 10 mM KMTB and 1.0, 2.5, 5.0, or 10.0 mM leucine.

All samples were analysed by pre-column derivatisation and reverse-phase HPLC. Ten µl of sample was mixed with 50 µl of 400 mM borate pH 10.5 and then with 10 µl of 10 mg/ml o-phthalaldehyde/12 µl/ml mercaptopropionate/400 mM borate pH 10.5 prior to the injection of 7.0 µl onto a 2.1 x 200 mm ODS-AA column (Agilent; Mississauga, ON, Canada). The column was eluted using 2.72 mg/ml sodium acetate pH 7.2/0.018% v/v triethylamine/0.3% v/v tetrahydrofuran as Buffer A and 2.72 mg/ml sodium acetate pH 7.2/40% v/v methanol/40% v/v acetontrile as Buffer B with a linear gradient of 0 – 17% B over 16 min followed by a linear gradient of 17-100% B over 1 min and 6.0 min at 100% B. The flow rate was 0.45 ml/min from 0 – 16 min and 0.80 ml/min from 17-30 min. The elution of derivatised amino acids was monitored at 331 nm. All separations were performed on an Agilent 1100 HPLC equipped with an autosampler, variable wavelength ultraviolet/visible spectrophotometric detector, and Chemstation operating system.

Cloning and Functional Expression

Genomic DNA was isolated from *B. subtilis* 168 by digestion with 0.3 % w/v lysozyme for 1 hr on ice, followed by incubation with an equal volume of 100mM NaCl/10 mM Tris-HCl pH 8.0/25 mM EDTA/0.5% w/v sodium dodecyl sulfate/0.1 mg/ml proteinase K at 37°C for 1 hr with occasional mixing. The mixture was then subjected to extraction with phenol and chloroform:isoamyl alcohol (24:1), and the DNA ethanol precipitated.

The nucleotide sequences of the *B. subtilis* aminotransferases were obtained from the SubtiList web site genolist.pasteur.fr/SubtiList/ [18] and used to design oligonucleotide primers for each enzyme (Table 1). The 5' primers contain a 12 nucleotide LIC (ligation independent cloning, [19]) sequence and an in-frame start codon, while the 3'primers contained a 13 nucleotide LIC sequence and an in-frame stop codon. The target sequences were amplified from the genomic DNA using Taq polymerase (Promega; Madison, WI,

USA), 1.5 mM $MgCl_2$, 200 µM dNTP, and the following program: 1 cycle of 95°C for 1.5 min, 30 cycles of 95°C for 1 min/55°C for 1 min/72°C for 1 min, and 1 cycle of 72°C for 10 min. The amplified target sequence was excised from a 1% agarose gel and the DNA extracted using the QiaexII kit (Qiagen;Mississauga, ON, Canada). The genes were then cloned into pCALnFLAG using the LIC procedure outlined by Stratagene (La Jolla, CA, USA), and then transformed into *E. coli* XL10 competent cells (Stratagene). The recombinant plasmid was purified from these cells using the QiaSpin miniprep kit (Qiagen), and the presence of the insert confirmed by digestion with NdeI and SacI and electrophoresis on a 1% agarose gel. The plasmid from positive clones was transformed into *E. coli* BL21 DE3 CodonPlus RIL cells (Stratagene) for functional expression.

The BL21 cells containing the recombinant plasmid were grown in LB liquid medium containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C and 250 rpm until the cell density reached an A_{600nm} of 0.6 – 0.8. The culture was then cooled to 28°C and IPTG added to 1.0 mM before 2-5 hr of continued culture at 28°C and 250 rpm. The cells were then pelleted by centrifugation at 3500 x g for 20 min at 4°C, and resuspended in a minimal volume of 10 mM mM HEPES pH 7.8/150 mM NaCl/1.0 mM DTT/1.0 mM imidazole/2.0 mM $CaCl_2$ before storage at –20°C. The sample was thawed, sonicated on ice, and centrifuged at 3500 x g for 20 min at 4°C. The resulting supernatant was loaded onto a 1.6 x 8.0 cm calmodulin-agarose column (Stratagene) equilibrated with the resuspension buffer. The column was eluted with 10 mM HEPES pH 7.8/1.2 M NaCl/1.0 DTT/3.0 EGTA. The eluted enzyme was concentrated to less than 5.0 ml using a 30 Kda molecular weight cut-off centrifugal filter (Pall Filtron; Mississauga, ON, Canada). The concentrated enzyme was kept at 4°C for short term storage and at –20°C with 20% v/v glycerol for long term storage.

Protein concentration was determined using the Bio-Rad dye (Mississauga, ON, Canada). Protein samples were examined by electrophoresis on 10% SDS polyacrylamide gels followed by Coomassie R250 staining.

Phylogenetic Analysis

Aminotransferase sequences were obtained from GenBank and were aligned using the Clustal algorithm and the BLOSUM sequence substitution table in the ClustalX program [20]. Aligned sequences were visualised with the Bioedit program [21].The aligned sequences were then used with the ProtDist component of Phylip [22] to construct a distance matrix which was the basis for tree construction using neighbor-joining [23]. All trees were visualised using Treeview [24].

Results

Methionine Regeneration in *B. subtilis* Homogenates

Subcellular homogenates of *B subtilis* were examined for the range of effective amino donors for the transamination of KMTB (Figure 2). Alanine, isoleucine, leucine, and valine were clearly the most effective amino donors, with phenylalanine and tyrosine also able to act as donors. The maximal rate of activity ranged from 1.20 – 1.48 nmol/min mg protein (for Ile:KMTB), which was equivalent to that seen in *K. pneumoniae* homogenates (1.92 nmol/min/mg protein for Glu:KMTB; [13]).

The homogenate was fractionated over three columns to partially purify the Met regeneration activity. One major fraction of activity was discovered which eluted at 300 mM KCl on a DEAE column, 280 mM PO_4 on hydroxylapatite, and 390 mM KCL on a mono-Q column. Analysis of the amino acid donor preference for Met production in this fraction demonstrated that alanine, valine, leucine, and isoleucine were the most effective substrates (Figure 2). Glutamate, phenylalanine, glutamine, and tyrosine were also able to act as amino donors at a lower rate.

B. subtilis AspATs are Members of the Aminotransferase If Subfamily

Previous research into the final step of Met recycling in the lower eukaryotes *C. fasciculate*, *T. brucei brucei*, *G. intestinalis*, and *P. falciparum*, and the gram-negative bacterium *K. pneumoniae* has demonstrated that the reaction in these organisms is catalysed by AspATs and TyrAT that belong to the Ia subfamily of the enzymes [11,13]. However, tree construction of the complete aminotransferase I family clearly showed that gram-positive bacteria and archaeabacteria had no enzymes in the Ia subfamily [13]. The previously assembled phylogenies have suggested that gram-positive bacterial AspATs are members of the aminotransferase If subfamily. The *B. subtilis* genome has six open reading frames (aspB, patA, ykrV, yugH, ywfG, yhdR) with significant homology to AspATs from diverse organisms [25]. One of these putative AspATs, the aspB gene product, corresponds to an AspAT which had been previously purified and characterised from cell homogenates [15]. Alignment of the six *B. subtilis* sequences with selected members of the aminotransferase I family, and construction of a phylogeny via neighbor-joining, clearly showed that the *B. subtilis* sequences clustered with other enzymes of the If subfamily (Figure 3). Therefore, the putative AspATs in *B. subtilis* are all members of the If subfamily.

Sequence alignment and tree construction of all the existing members of the If subfamily available in public databases showed that the *B. subtilis* sequences are broadly dispersed among several divisions of the subfamily (Figure 4). The yhdR sequence was 50% identical to the *B. halodurans* BH2195 gene product and had a low identity to the other subfamily If enzymes. The ykrV and ywfG sequences were 49% identical to each other, and had a 60% and 48% identity to the *B. halodurans* BH1060 gene product. The two sequences also had a respective 40% and 37% identity to the *Staphylococcus aureus* SAV2560 gene product. The

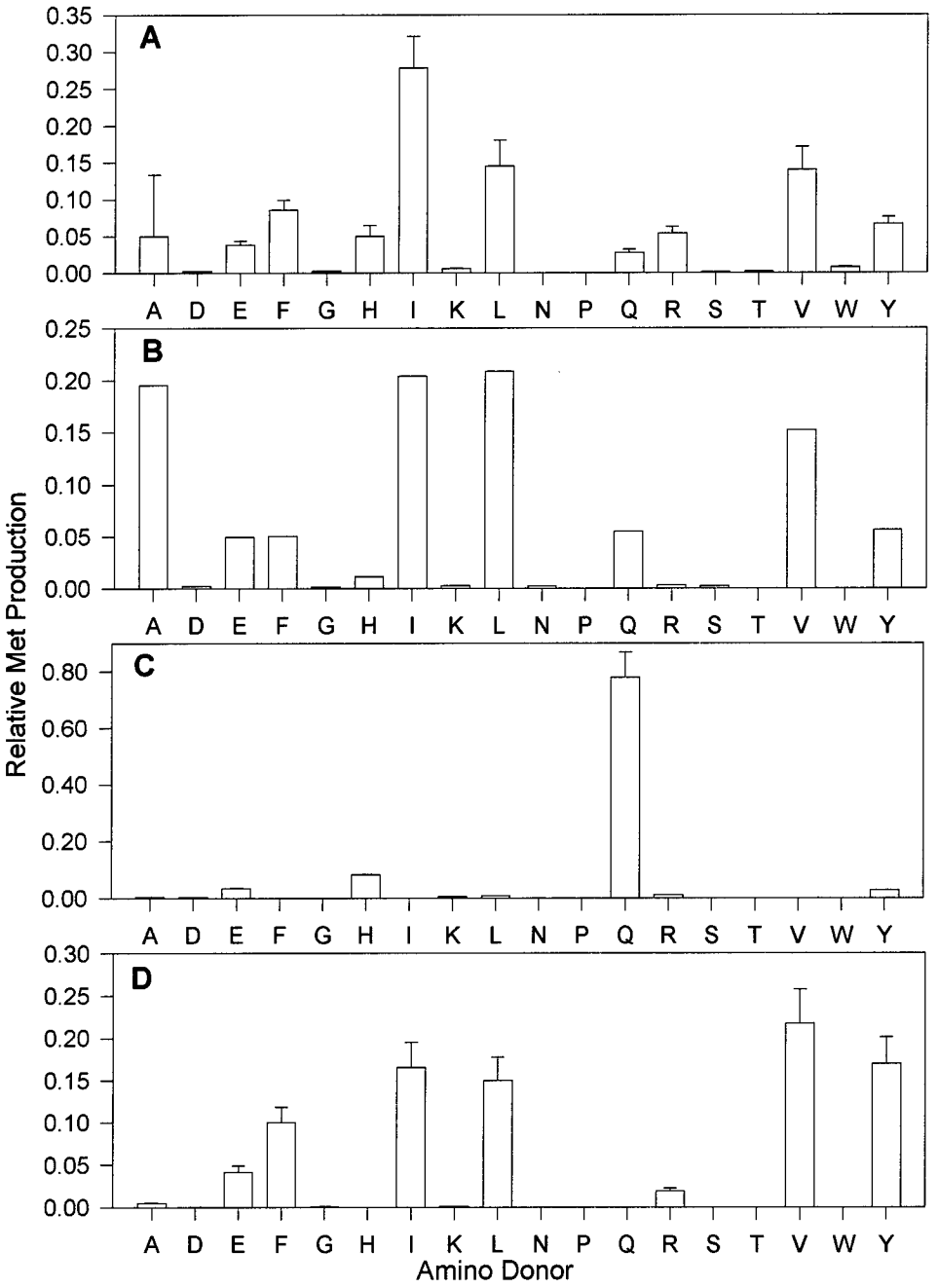

Figure 2. *The amino donor range for Met regeneration in B. subtilis. An enzyme source was mixed with 1.0 mM KMTB, 2.0 mM of an individual amino acid, and pyridoxal phosphate for 30 min at 37°C before analysis of Met production by HPLC. The enzyme sources are: (A) B. subtilis homogenate, (B) semipurified fraction from B. subtilis homogenate, (C) recombinant ykrV gene product, and (D) recombinant ybgE gene product.*

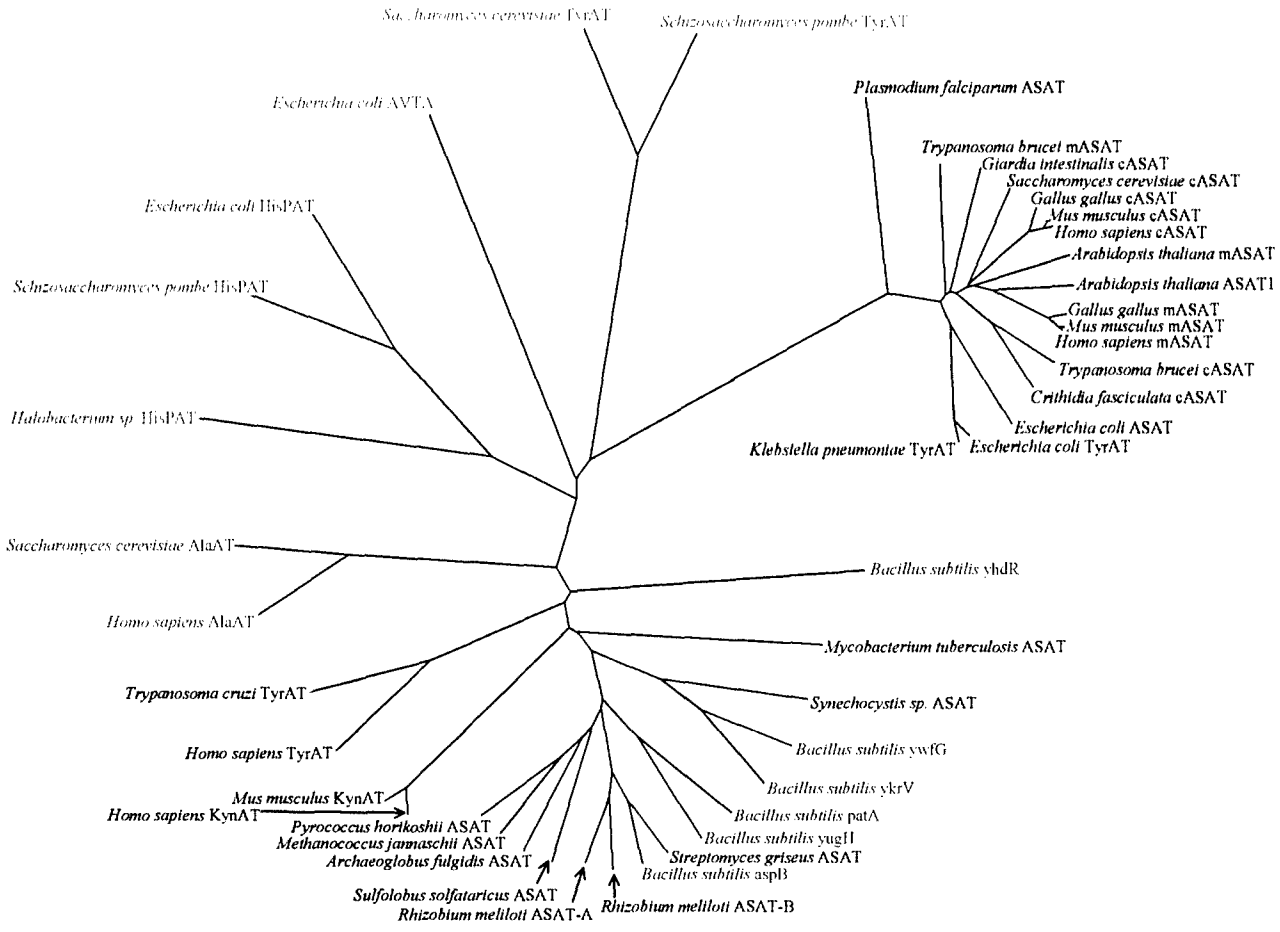

Figure 3. *Family I aminotransferases. Selected sequences were aligned via the clustal algorithm and utilised for tree construction with the neighbor-joining method. The B. subtilis sequences are in red, known members of amnotransferase subfamily Ia in blue, Ib in green, Ie in purple, If in black, Ig in pink, and Ih in orange. The enzymes are abbreviated: ASAT, aspartate aminotransferase; TyrAT, tyrosine aminotransferase; AVTA, alanine:valine aminotransferase; HisPAT, histidinol-phosphate aminotransferase; AlaAT, alanine aminotransferase; KynAT, kynurenine aminotransferase.*

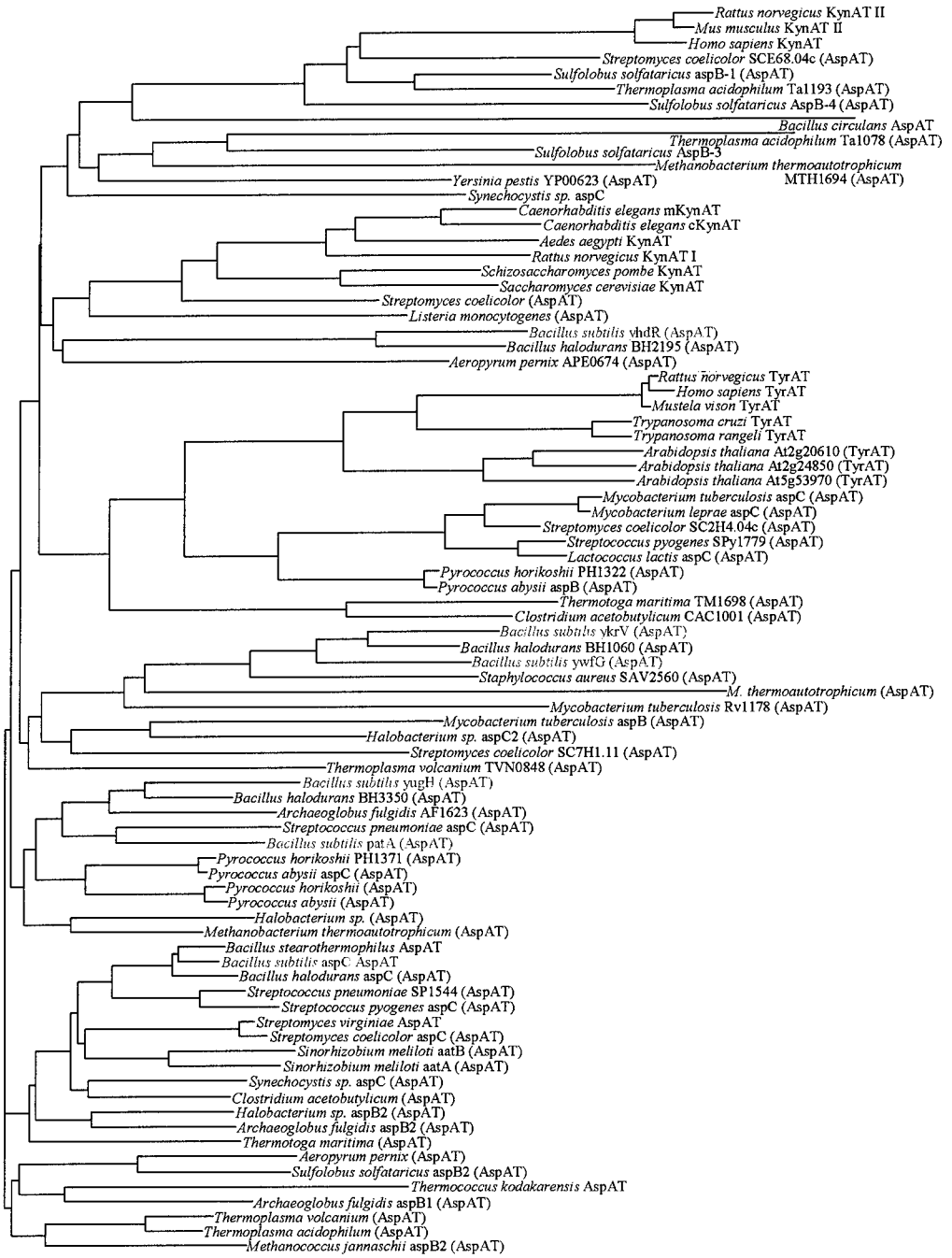

Figure 4. *The If subfamily of aminotransferases. The sequences were aligned with the clustal algorithm and used for tree construction with the neighbor-joining method. The putative B. subtilis AspATs are in red.*

yugH sequence was 53% identical to the *B. halodurans* BH3350 gene product, 42% to the *Archaeoglobus fulgidis* AF1623 gene product, and 34% to the *B. subtilis* patA sequence. This latter sequence was 45% identical to the *Streptococcus pneumoniae* AspC gene product. Finally, the *B. subtilis* aspB sequence was 73% identical to the *B. halodurans* aspC gene product and 79% to the *B. stearothermophilus* aspC gene product. It is interesting to note that, aside from the ykrV and ywfG sequences, the putative *B. subtilis* AspATs bear little resemblance to each other and are highly divergent within subfamily If.

Characterisation of the Putative *B. subtilis* AspATs

Alignment of the six *B. subtilis* sequences with selected subfamily If enzymes that have been fully characterised in the literature highlighted the lack of sequence conservation within subfamily If (Figure 5). In the alignment, only Y115, G120, Y181, P238, N240, P241, G243, D274, Y277, K311, G317, R401, and R463 are conserved across the twelve enzymes. Of these residues, G243(G197)*, D274(D222), K311(K258), and R463(R386) are reported by Mehta et al. to be conserved across all four aminotransferase families [26]. Residues Y115(Y70), G120(G110), N240(N194), P241(P195), Y277(Y225), and G317(G268) are reported by Jensen and Gu to be conserved across all family I aminotransferases [14]. Therefore, unlike subfamily Ia aminotransferases, the subfamily If enzymes do not present a recogniseable unique sequence motif.

The aspB, patA, yugH, ykrV, and ywfG genes were cloned and functionally expressed as calmodulin-binding peptide fusion proteins. Using the primer sequences listed in Table 1, which exactly match the sequence determined in the *B. subtilis* genome project, the yhdR gene could not be amplified. The basis for this lack of amplification is currently being further investigated. The five recombinant proteins were purified by affinity chromatography over a calmodulin-agarose column (Figure 6), where all except the yugH gene product yielded large amounts of soluble enzyme. In the case of yugH, the majority of the expressed protein was recovered as insoluble inclusion bodies. Never the less, sufficient soluble yugH fusion protein was recovered to allow for characterisation of activity.

Each of the putative enzymes was screened for Met regeneration activity using a mixture containing 1.0 mM KMTB as amino acceptor and 2.0 mM ADEFGHIKLNQRSTWY as potential amino donors. The yugh, ywfG, patA, and aspB gene products catalysed little detectable Met regeneration under these conditions. Only the ykrV gene product was able to produce a moderate amount of Met (approximately 40 nmol/min/mg protein) using the mixture of amino donors. The enzyme was rescreened using 1.0 mM KMTB and 2.0 mM of each individual amino acid as amino donor (Figure 2). The enzyme was found to have an almost exclusive preference for Gln as an amino donor for the transamination of KMTB. The enzyme was also found to be similarly active in catalysing Gln:KG aminotransfer (data not shown). In this regard, the ykrV gene product has the characteristics of a glutamine aminotransferase (KynAT).

* By the convention of Mehta et al. [26], conserved residues are also labelled in parentheses with the corresponding residue number from pig cytosolic AspAT.

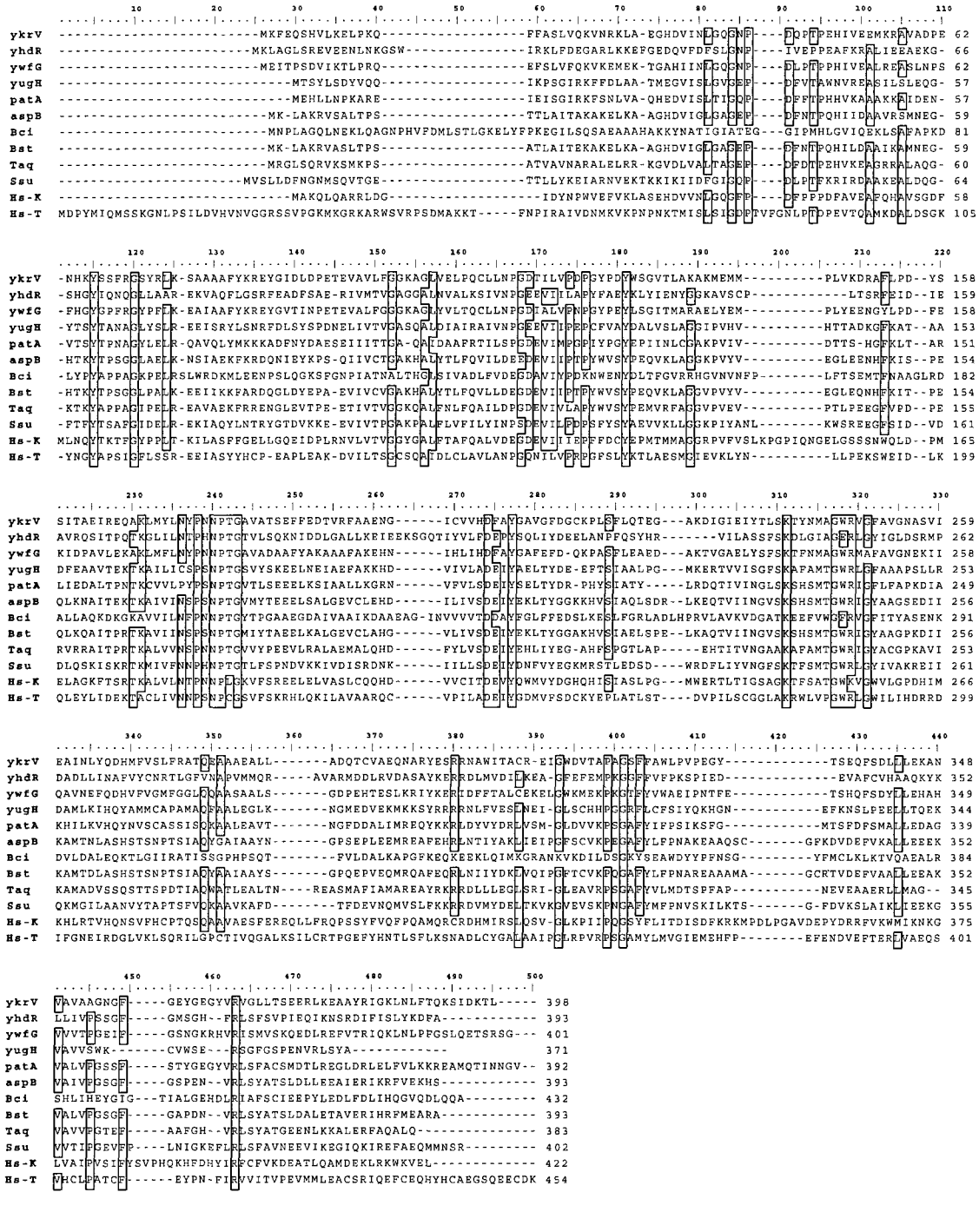

Figure 5. *Alignment of the putative B. subtilis AspATs. The following sequences were aligned using the clustal algorithm: B. subtilis ykrV, yhdR, ywfG, yugH, patA, aspB, (Bci) B. circulans AspAT [27], (Bst) B. sp. AspAT [28], (Taq) Thermus aquaticus AspAT [29], (Ssu) Sulfolobus sulfotaricus AspAT [16], (Hs-K) human KynAT [30], and (Hs-T) human TyrAT [31]. Residues conserved by 75% of the sequences are highlighted.*

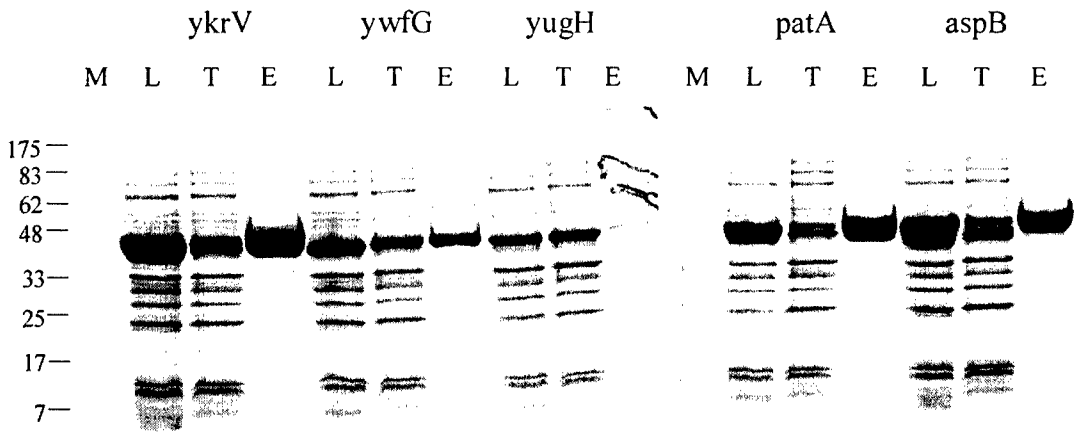

Figure 6. *Purification of recombinant B. subtilis AspATs. E. coli BL21 codon-plus cells (Stratagene) carrying the indicated transgenes were induced with IPTG and prepared as described in the Materials and Methods section. The clarified homogenate (L) was loaded onto a calmodulin-agarose column, and the flow-through (T) and eluates (E) collected. Aliquots of each fraction were analysed on 10% acrylamide gels under reducing conditions. Lane (M) contains molecular weight markers.*

Of the putative *B. subtilis* AspATs, only the ykrV gene product was effective at transaminating KMTB. However, the amino donor specificity for recombinant ykrV did not match the major activity seen in *B. subtilis* cell homogenates or the corresponding semi-purified fraction. Another aminotransferase(s) is clearly responsible for the cellular activity seen using branched-chain and aromatic amino acids as amino donors.

Characterisation of Putative *B. subtilis* BCATs

The strong preference for branched-chain amino acids as amino donors suggested the possibility that a branched-chain amino acid aminotransferase (BCAT) was responsible for this Met regeneration activity in *B. subtilis*. In a previous publication, Hall et al. [32] have found that rat brain BCAT could catalyse Met:KG aminotransfer at a rate approximately 10% of Leu:KG, but this reaction has not been further examined, particularly in the reverse reaction. Similarly, bacterial BCATs are known to play a role in the conversion of Met to odour compounds in cheese bacteria [33,34], but use of KMTB in the reverse reaction has not been studied.

BCATs are members of family III of the aminotransferase superfamily, and appear to be evolutionarily unrelated to the family I and II aminotransferases [35]. Members of family III have not been subjected to the same level of phylogenic analysis as family I, and there is no information available on potential subfamilies within family III. Clustal alignment of all the available family III aminotransferase sequences followed by tree construction using a variety of algorithms demonstrated a clear division into two subfamilies (Figure 7). The first, designated subfamily IIIa, contained BCATs from eukaryotic and bacterial sources. The second, designated subfamily IIIb, contained BCATs from archaeal and bacterial sources, as well as D-amino acid aminotransferases (DAATs). Analysis of the *B. subtilis* genome data [25], has uncovered three sequences with homology to existing members of family III: ywaA, ybgE, and yheM (Figure 7). The yheM sequence clearly localised amongst the DAAT sequences in subfamily IIIb, and was 62% identical to the *B. licheniformis* DAAT and 45% identical to the *Staphylococcus haemolyticus* DAAT. The ybgE and ywaA sequences were found in the IIIa subfamily, and were 59% identical to each other. The ybgE sequence was also 60% identical to the *B. halodurans* BAB05575 gene product and 40% identical to the *Xylella fastidiosa* XF1999 gene product. The ywaA sequence was 62% and 42% identical to these sequences.

Alignment of yheM, ybgE, and ywaA with seven other family III sequences that have been fully characterised in the literature demonstrated a low number of conserved residues across the family (Figure 8). Residues E104(E37)*, F119(F51), R127(R59), K233(K159), N237(N163), G252(G178), E269(E193), T290(T209), L298(L217), E342(E251), P354(P263), and G376(G278) are conserved across these ten sequences, with K233(K159) as the

* As the family III aminotransferases are now thought of as unrelated to the family I and II enzymes [35], it would be inappropriate to continue with the use of pig cytosolic AspAT as the reference sequence for family III enzymes. The residues in parentheses reflect the corresponding residue in the *E. coli* ilvE gene product, which has been well studied in the literature.

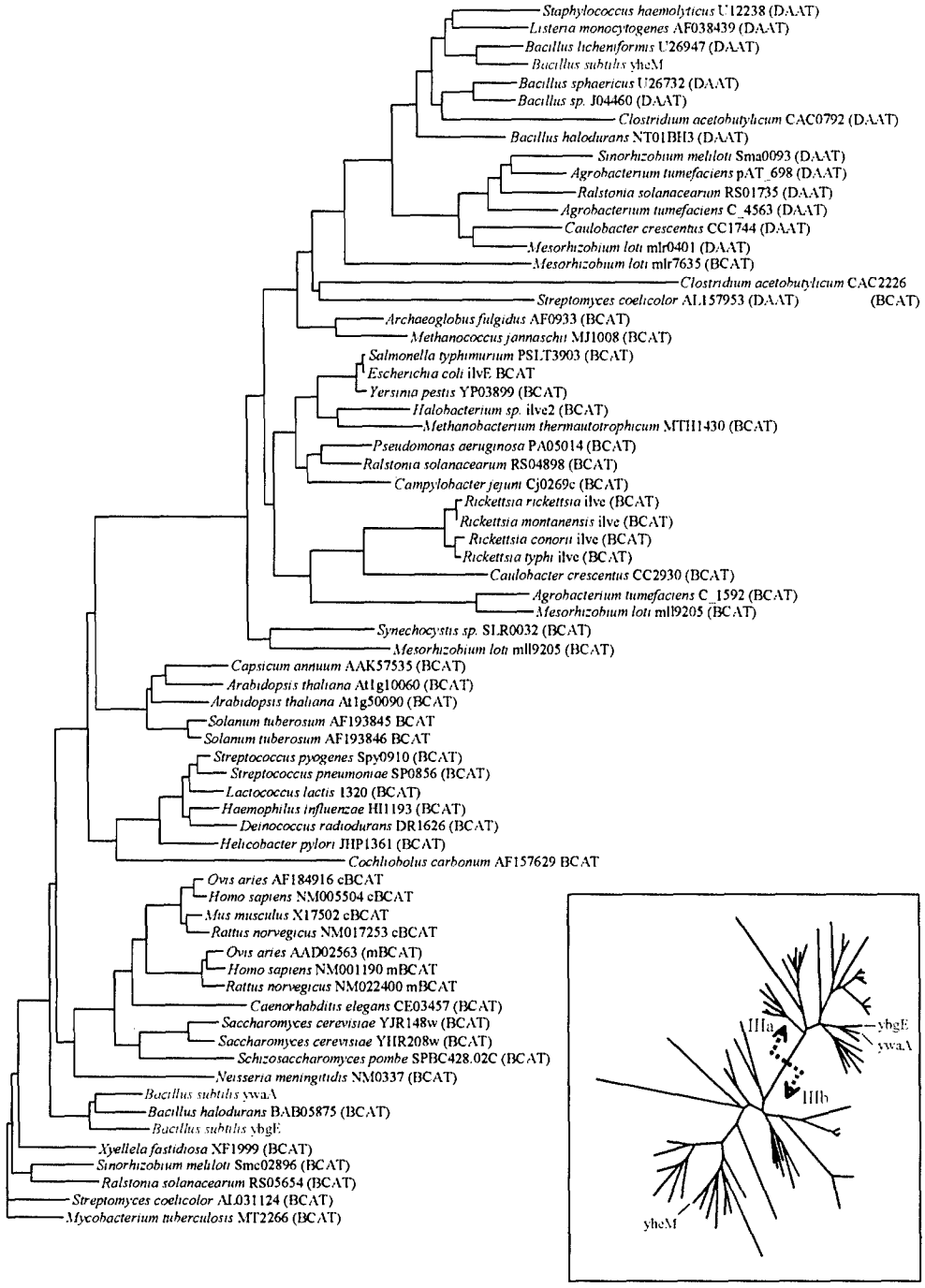

Figure 7. *Family III aminotransferases. The sequences were aligned with the clustal algorithm and used for tree construction with the neighbor-joining method. The putative B. subtilis family III sequences are in red. The inset presents the same data as an unrooted tree, with the two proposed subfamilies labelled in blue.*

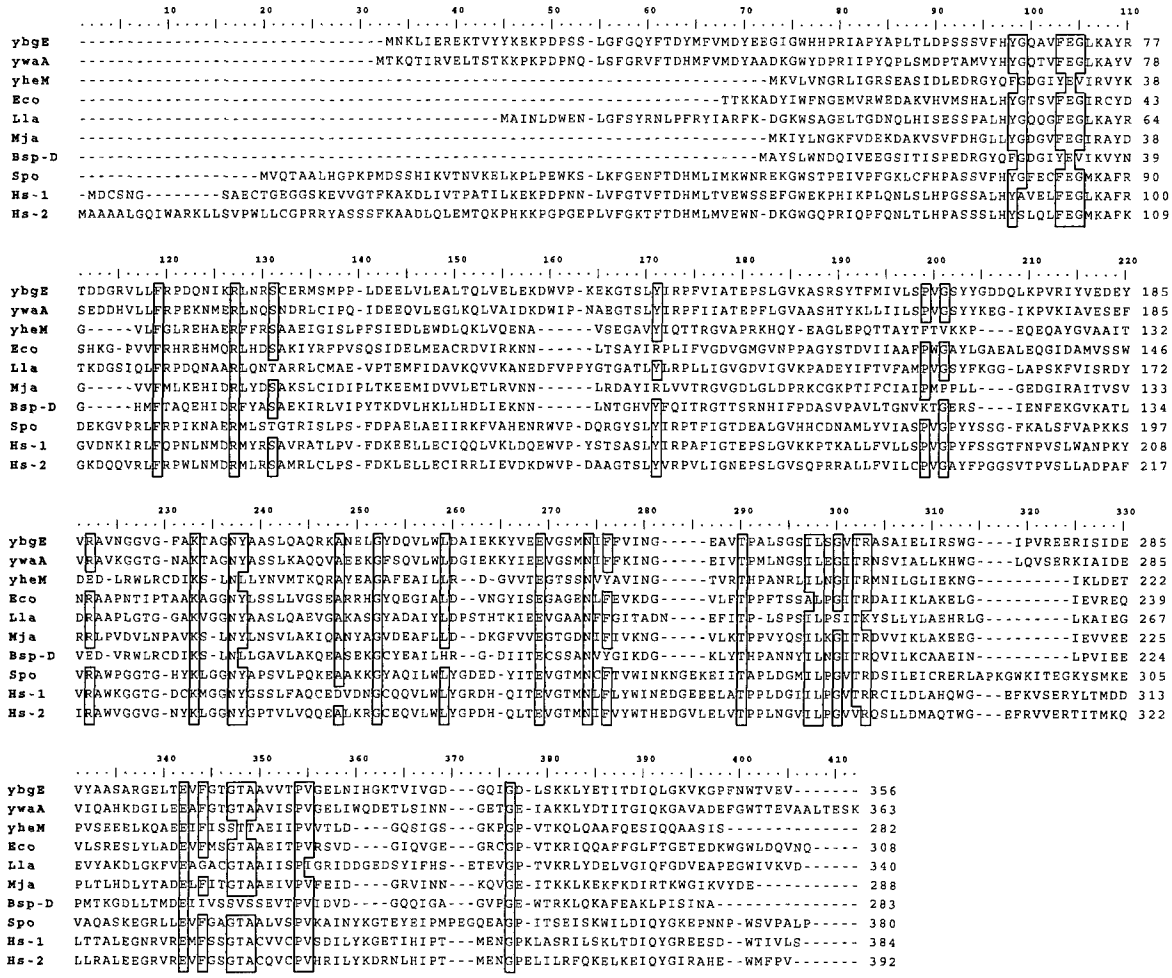

Figure 8. *Alignment of the putative B. subtilis family III aminotransferases. The following sequences were aligned using the clustal algorithm: B. subtilis yheM, ybgE, ywaA, (Eco) E. coli BCAT [36], (Lla) Lactococcus lactis BCAT [33], (Mja) Methanococcus jannaschii BCAT [37,38], (Bsp-D) Bacillus sphaericus DAAT [39], (Spo) Schizosaccharomyces pombe BCAT [40], (Hs-1) human BCAT-1 [41], and (Hs-2) human BCAT-2 [42]. Residues conserved by 75% of the sequences are highlighted.*

pyridoxal-phosphate binding site. Even amongst this small selection of family III enzymes it is clear that there is no retention of D/E222 (in pig cytosolic AspAT) postulated by Mehta et al. [26] as an invariant residue across all aminotransferases. While completely conserved across family I and II enzymes, this residue appears to be unneccessary for activity in family III enzymes. A more detailed alignment of each family III subfamily and the presence of sequence motives will be discussed elsewhere.

The two putative BCATs were cloned and functionally expressed as calmodulin-binding peptide fusion proteins. The ybgE gene product was almost completely soluble, while that from ywaA was about 75% insoluble as inclusion bodies (Figure 9). Both recombinant enzymes were screened using KMTB and single amino acids as amino sources. The ywaA gene product catalysed little Met production, whereas the ybgE gene product readily formed Met using leucine, isoleucine, valine, phenylalanine, and tyrosine as amino donors (Figure 2). Both enzymes were active in leucine:KG aminotransfer (data not shown).

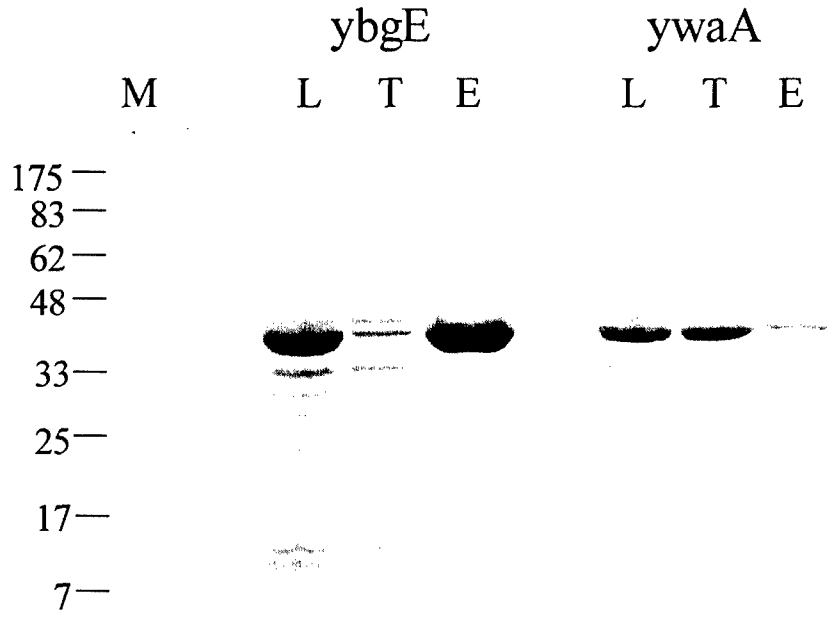

Figure 9. *Purification of recombinant B. subtilis BCATs. E. coli BL21 codon-plus cells (Stratagene) carrying the indicated transgenes were induced with IPTG and prepared as described in the Materials and Methods section. The clarified homogenate (L) was loaded onto a calmodulin-agarose column, and the flow-through (T) and eluates (E) collected. Aliquots of each fraction were analysed on 10% acrylamide gels under reducing conditions. Lane (M) contains molecular weight markers.*

As the amino donor preference for Met regeneration with the recombinant ybgE closely mirrored that seen in B. subtilis homogenates and in the semipurified fraction, more detailed kinetic studies were performed on the BCATs (Table 2). The Km values for Leu, Val, and Ile in the presence of KMTB or KG were very similar for both ybgE and ywaA, and ranged from 2.11 – 5.68 mM. Both ybgE and ywaA catalysed KMTB transamination at a rate

approximately 10-fold lower than the corresponding reaction with KG. In addition, ywaA was 100-fold less active than ybgE for all of the reactions examined. Therefore, it would appear that the ybgE gene product is responsible for the majority of the Met regeneration seen in the *B. subtilis* homogenates.

Inhibition of the ybgE Gene Product with Canaline

The production of Met from KMTB and leucine was examined in the presence of the aminotransferase inhibitor canaline, which has been demonstrated as an effective inhibitor of Met regeneration [10-13]. Using 10 mM KMTB, 1.0 - 10 mM leucine, and 0 – 1.0 mM canaline, the inhibitor was found to interfere with the reaction in an uncompetitive manner (Figure 10). The calculated Ki was found to be 48.41 ± 19.64 µM canaline.

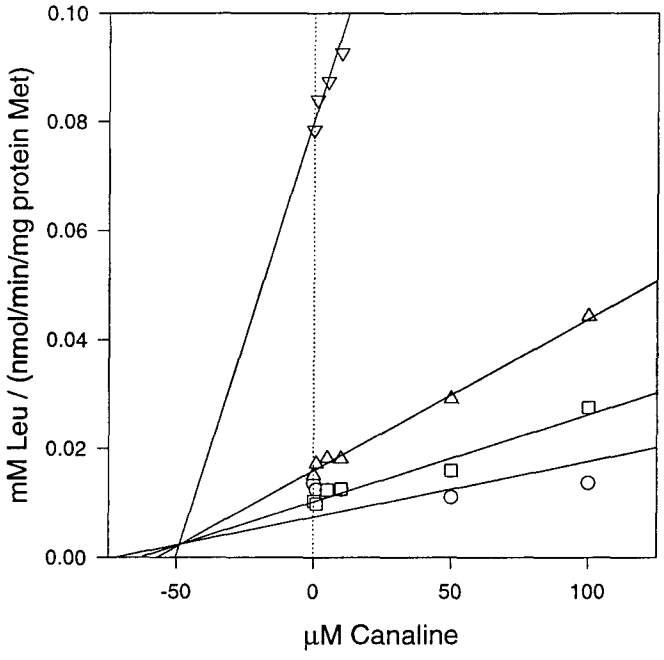

Figure 10. *Inhibition of the ybgE gene product by canaline. Varying amounts of canaline were incubated with 10 mM KMTB and 1.0 (inverted triangles), 2.5 (triangles), 5.0 (squares), or 10.0 (circles) mM leucine before quantitation of Met production by HPLC.*

Canaline was also examined as an inhibitor of *B. subtilis* growth in vitro. When 10^4 cfu of *B. subtilis* were used as the inoculum in a final volume of 200 µl of nutrient broth, up to 5.0 mM canaline was found to have little effect on cell growth over 24 hours (Figure 11). When the experiment was repeated using a minimal medium, canaline was found to be an effective growth inhibitor, with an IC_{50} of 36.69 ± 9.62 µM and an MIC of 500 µM (Figure 11).

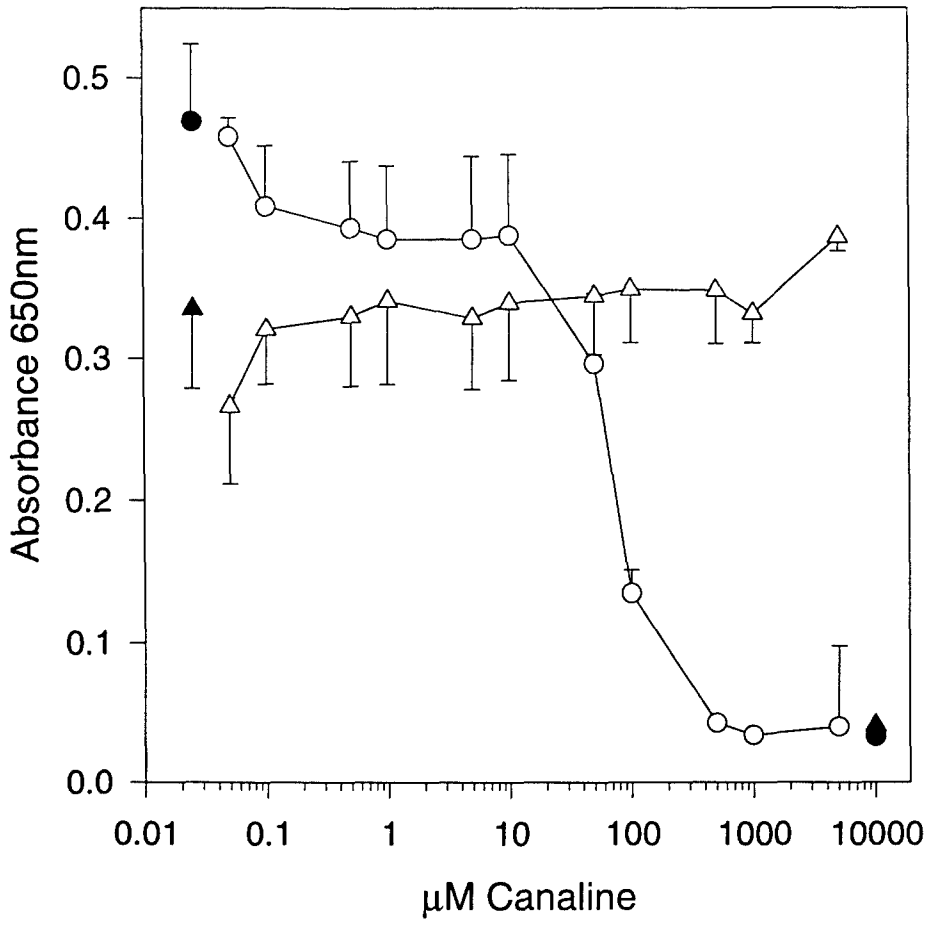

Figure 11. *In vitro inhibition of B. subtilis growth by canaline. 2 x 10^4 cfu of B. subtilis early log cells were inoculated into nutrient broth (triangles) or minimal medium (circles) in the presence of varying concentrations of canaline. Growth after incubation overnight at 30°C was measured by turbidity at 650 nm. The dark circles and triangles are the appropriate values for growth with no inhibitor and for medium without cells.*

Discussion

Polyamine biosynthesis, and its associated Met regeneration pathway, have been the subject of a number of studies on experimental chemotherapeutics in bacteria, protozoa, and cancer cells [1]. To date, none of the previous reports have examined this potential in gram-positive bacteria. Many of the enzymes shown in Figure 1 have been characterised in *B. subtilis*, including S-adenosylmethionine synthase [43], S-adenosylmethionine decarboxylase [44], methylthioadenosine nucleosidase [45], and methylthioribose kinase [46], but have not been considered as drug targets. The existence of a complete genome sequence for *B. subtilis* 168 facilitates the use of this organism as a model for the development of inhibitors against *B. cereus* and *B. anthracis* [25]. Our previous work in parasitic protozoa and *K. pneumoniae* had identified the regeneration of Met from KMTB as a potential target for therapeutic development [10,11,13]. In all previous organisms examined, this reaction was catalyzed by an aminotransferase from the Ia subfamily of the enzymes. In this paper, we have demonstrated that *B. subtilis* has no aminotransferase sequences which fall within the Ia subfamily. Instead, the analogous enzymes in *B. subtilis* are all members of the If subfamily. These enzymes have been cloned, expressed, and characterised, and found not to be the main catalysts of Met regeneration.

Further examination of *B. subtilis* homogenates, and partial purification of the Met recycling activity implicated an aminotransferase with a preference for utilising branched-chain amino acids as the amino donors for KMTB (Figure 2). The two genes identified from the *B. subtilis* genome data as having high identity with BCATs were cloned, expressed, and characterised. One of these enzymes, the ywaA gene product, was found to catalyze Met formation using the same amino donor preference seen in sub-cellular homogenates. We are therefore able to identify the ybgE BCAT as the primary source of Met recycling in *B. subtilis*.

Unlike the situation seen with the TyrAT in *K. pneumoniae*, where tyrosine:KMTB and tyrosine:KG aminotransfer occurred at equal rates [13], the *B. subtilis* ywaA gene product catalyzes leucine:KG aminotransfer approximately 10-fold better than leucine:KMTB aminotransfer. When growing in nutrient broth, *B. subtilis* has access to large amounts of exogenous Met and might repress the expression of an aminotransferase required for Met regeneration. However, we have examined subcellular homogenates made from *B. subtilis* grown in minimal medium with no exogenous Met and sulfate as the sulfur source, and have found no substantial increase in the formation of Met from KMTB (data not shown).

That family III aminotransferases might catalyze Met regeneration in the place of subfamily Ia aminotransferases is interesting. Family I and family III enzymes appear to be unrelated and the result of convergent, rather than divergent, evolution. Structural studies have shown that family I (and family II) aminotransferases consist of different folds, and do not have a spatial resemblance [35]. In addition, family III enzymes are substantially smaller than family I aminotransferases and lack one of the four universally conserved residues found in the other aminotransferase families [26]. The evolution of this functionality in family III aminotransferases in *B. subtilis* suggests that this family of enzymes should be examined in other Gram-positive bacteria and archaeabacteria, which lack subfamily Ia enzymes, and in mammals, where the subfamily Ia enzymes have been found not to catalyse Met regeneration

[10]. The work of Hutson et al. [47] have shown that rat and human BCAT can catalyze Met:KG aminotransfer, but this finding has not been further developed.

Amongst the subfamily If enzymes examined in this paper, only one, the ykrV gene product, was found to have any appreciable Met regeneration activity. However, this enzyme only produced Met from KMTB using glutamine as an amino donor, did not have any aspartate:KG activity, and had substantial glutamine:KG activity. This profile did not match with that seen in subcellular homogenates or in the partially purified Met recycling activity (Fig 2). Recently, Sekowska et al. [46] have identified members of the ykrTS and ykrWXYZ operons as enzymes involved in Met regeneration. These operons were found to be members of the S-box regulon [48], which is up-regulated during sulfur limitation. In particular, the authors have unequivocally identified the ykrT gene product as the *B. subtilis* methylthioribose kinase (see Figure 1). By gene deletion, the ykrS gene product has also been identified as essential for Met regeneration from methylthioribose. The other members of the operon are implicated by association as being involved in Met recycling, but have not been definitively proven to participate. The ykrV gene product lies immediately between the ykrTS and ykrWXYZ operons and could be thought to act as the catalyst for the last step in the cycle. However, while we have shown that the ykrV gene product will convert KMTB to Met using glutamine as an amino donor, it does not act in this capacity under the conditions we have examined. Perhaps ykrV plays a role in Met recycling under specific growth conditions not replicated by planktonic growth in nutrient broth or minimal medium. This aspect of ykrV function deserves further attention.

As seen with the other enzymes previously investigated [10,11,13], canaline acts as an efficient inhibitor of Met regeneration by the ybgE gene product with a Ki of 48 μM. However, in nutrient broth, *B. subtilis* growth in the presence of 5.0 mM canaline showed little inhibition. In a minimal medium containing no exogenous Met, cysteine, or protein, canaline was an effective inhibitor of *B. subtilis* growth with an IC50 of 37 μM and an MIC of 500 μM. It is interesting to note that the IC50 for canaline in this minimal medium is very similar to the Ki for canaline against ybgE, suggesting that inhibition of this enzyme plays a role in the toxic effect of the drug. The lack of effect of canaline against *B. subtilis* in nutrient broth is possibly due to antagonism by exogenous Met or binding of canaline to exogenous protein. Experiments are currently underway to define the lack of activity in nutrient broth and its potential implications for canaline activity under physiological conditions.

These studies were undertaken using *B. subtilis* as a biochemical model for *B. anthracis* due to the fact that *B. subtilis* has a completed genome sequence and is also non-pathogenic. Indeed, *B. subtilis* has been used regularly as a spore simulant for *B. anthracis* [49,50]. During the course of this work, a >95% complete genome sequence was publicly released for *B. cereus* (www.integratedgenomics.com). In addition, a genome project for *B. anthracis* is nearing completion (www.tigr.org). Therefore, due to the nearly complete genetic identity of *B. cereus* with *B. anthracis* and the relatively low pathogenicity of *B. cereus*, this organism will become the future focus of model biochemical studies in this laboratory.

Table 1. *Oligonucleotide primers used for amplification of the genes in this study. Primers in both directions contain 5' sequence complementary to the ligation-independent cloning site of pCALnFLAG (Stratagene).*

GENE		SEQUENCE
ykrV	5'	GACGACGACAAGATGAAATTTGAACAGTCTCATGTA
	3'	GGAACAAGACCCGTTTATAAGGTCTTGTCAA
ywfG	5'	GACGACGACAAGATGGAAATAACACCGTCCGATGTCA
	3'	GGAACAAGACCCGTTTAGCGGGATGTTTCTTGTAA
yhdR	5'	GACGACGACAAGATGAAATTGGCTGGGTTAT
	3'	GGAACAAGACCCGTTTAGCGGGATGTTTCT
yugH	5'	GACGACGACAAGATGACTTCGTATTTATCA
	3'	GGAACAAGACCCGTTCACCGCTCGGACCAAA
patA	5'	GACGACGACAAGATGGAACATTTGCTGAAT
	3'	GGAACAAGACCCGTTTAAACGCCGTTGTTTA
aspB	5'	GACGACGACAAGATGAAACTGGCAAAAAGAGTA
	3'	GGAACAAGACCCGTTTAGCTATGTTTTTCTACA
ybgE	5'	GACGACGACAAGATGAATAAGCTTATTGAACGAGAAA
	3'	GGAACAAGACCCGTTCACACTTCCACTGTCCAGTTAA
ywaA	5'	GACGACGACAAGATGACTAAACAAACAATTCGCGTTGA
	3'	GGAACAAGACCCGTTTACTTGCTTTCAGTCAGCGCTGCG

Table 2. *Kinetic characterisation of the Bacillus subtilis BCATs. The enzymes were incubated with varying amounts of substrate and 10 mM cosubstrate before analysis by HPLC as described in the Materials and Methods section.*

GENE PRODUCT	SUBSTRATE	COSUBSTRATE	APPARENT Km (mM)	APPARENT Vmax (nmol/min/mg protein)
ybgE	Leu	KG	3.99 ± 0.75	$1.46 \pm 0.12 \times 10^4$
	Val	KG	2.82 ± 1.55	$1.39 \pm 0.29 \times 10^4$
	Ile	KG	3.15 ± 0.93	$1.66 \pm 0.20 \times 10^4$
	Leu	KMTB	2.78 ± 0.76	$1.87 \pm 0.14 \times 10^3$
	Val	KMTB	3.20 ± 0.58	$2.03 \pm 0.09 \times 10^3$
	Ile	KMTB	2.36 ± 0.59	$1.84 \pm 0.12 \times 10^3$
ywaA	Leu	KG	3.04 ± 0.33	103.09 ± 26.27
	Val	KG	3.34 ± 1.22	48.49 ± 7.33
	Ile	KG	4.83 ± 1.33	226.68 ± 31.81
	Leu	KMTB	5.68 ± 0.39	21.03 ± 0.67
	Val	KMTB	ND	ND
	Ile	KMTB	2.11 ± 0.94	15.14 ± 2.36

ND = No detectable Met production

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List of symbols/abbreviations/acronyms/initialisms

Met	methionine
KMTB	ketomethiobutyrate
KG	ketoglutarate
AspAT	aspartate aminotransferase
TyrAT	tyrosine aminotransferase
BCAT	branched-chain amino acid aminotransferase
DAAT	D-amino acid aminotransferase
KynAT	kynurenine aminotransferase

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The aminotransferases involved in the final step of methionine recycling from methylthioadenosine have been examined in the gram-positive bacterium *Bacillus subtilis*. Homogenates of this bacterium were able to convert ketomethiobutyrate to methionine, utilising leucine, isoleucine, valine, phenylalanine, tyrosine, and alanine as preferred amino donors. Unlike other organisms examined to date in this context, *B. subtilis* was found to contain no aspartate aminotransferase or tyrosine aminotransferase sequences with structural homology to subfamily Ia aminotransferases. Instead, in *B. subtilis*, the six putative homologues of aspartate aminotransferase were found to be members of the If subfamily. Five of these six sequences were cloned and expressed, with only the ykrV gene product capable of producing methionine from ketomethiobutyrate. However, this enzyme only catalysed the reaction using glutamine as an amino donor. Two putative branched-chain amino acid aminotransferases from family III were also cloned and expressed, and both were found to produce methionine from ketomethiobutyrate using branched-chain and aromatic amino acids. Of these two enzymes, the ybgE gene product was the most active and had kinetic constants consistent with it being the enzyme responsible for the majority of methionine regeration in this organism. The ybgE gene product could be inhibited uncompetatively by the aminooxy compound canaline, with a Ki of 48 μM. In addition, canaline inhibited the in vitro growth of *B. subtilis* in minimal medium with an IC_{50} of 37 μM and an MIC of 500 μM. Growth inhibition in nutrient broth was negligible.

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Bacillus subtilis, methionine recycling, aminotransferases, inhibition, canaline

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